Summary

This thesis describes the molecular genetic analysis of two alleles of the *Amf* locus of potato: a wild-type allele and the *amf* allele derived from it. Other aspects of the analysis of the *Amf* locus concern the expression of homologous genes introduced via *Agrobacterium rhizogenes*, the synthesis and accumulation of amylose, chimerism, intracellular transport of proteins, and the analysis of the structure of a gene adjacent to the *Amf* locus.

Chapter I gives a brief introduction to some of the genes and enzymes involved in starch synthesis, some background information about the amylose-free (amf) potato mutant and protein transport into plastids, and an outline of this thesis.

Chapter II describes the experiments which provided conclusive evidence that the *Amf* locus in potato is identical with the structural gene for granule-bound starch synthase (GBSS). By introducing a wild-type allele of the GBSS gene into the amylose-free mutant, the accumulation of amylose in starch could be restored.

Gene transfer was carried out with the use of a binary *Agrobacterium rhizogenes* system, which accomplishes the stable integration of vector DNA in the potato genome. Transgenic hairy roots can be obtained within 10 days after inoculation of stem segments with *A. rhizogenes*. These hairy roots contain in their tips, like normal roots, columella cells with starch. In wild-type plants the starch stains blue with Lugol's solution (I_2/KI), but in the amf mutant the starch stains red. After the inoculation of amf stem segments with an *A. rhizogenes* strain carrying a plasmid with the GBSS gene next to a kanamycin resistance gene, a number of hairy roots contained starch staining blue with iodine. Transformation with a plasmid without the GBSS gene did not result in the formation of blue-staining roots.

To confirm that GBSS gene expression was restored we regenerated, via a callus phase, complete plants. Nearly 90% of the regenerated kanamycin resistant plants showed the presence of amylose. In tuber starch of complemented plants the GBSS activity was indeed restored and the amylose percentage did not significantly deviate from the percentage in wild-type potato tuber starch. That GBSS is not the sole regulating factor in amylose synthesis was confirmed by the observation that there was no positive correlation between the amount of GBSS activity and the percentage of amylose.
Examination of starch composition in different parts of the regenerated plants made clear that expression of a homologous gene is not always stable.

In an addendum to chapter II, an unexpected phenomenon after the introduction of the GBSS gene in wild-type plants is described. Some of the transformants showed amylose-free tubers: both the endogenous and the introduced GBSS gene were not expressed. The instability of gene expression in these so-called "co-suppressed" plants might be related to some of the phenomena found in complemented transformants of the mutant.

In chapter III the *Amf* and *amf* alleles of the GBSS gene are characterized and compared. The DNA and cDNA sequence analysis of the wild-type allele is given, together with a comparison of the deduced protein with the three sequences known at that time of waxy proteins from maize, barley and rice, all being highly similar. The mature parts of the GBSS proteins are homologous to the bacterial glycogen synthase glgA. The transit peptides, which are needed for the transport of these proteins into the plastids, differ at the primary sequence level, but show similarity in the distribution of hydrophilic and hydrophobic regions.

The *amf* allele was isolated and compared to the wild-type allele. Introduction of chimeric gene constructs enabled the localization of the *amf* mutation, and sequencing of the relevant part revealed that the mutation resides in the part coding for the transit peptide (TP). The DNA sequence of the *amf* allele differs from the sequence of the wild-type allele by one single nucleotide. A deletion of

an AT basepair, which means a shift in the reading frame, is the cause of the absence of amylose in the starch of the amf mutant. The frameshift results in the termination of translation at codon number 18 of the *amf* sequence. Since a GBSS-like protein, larger than mature GBSS and smaller than pre-GBSS, has been found in a membrane preparation of amf tubers, the hypothesis was put forward that translation re-initiates at an in-frame AUG codon, i.e., codon number 60 of the *Amf* sequence.

In chapter IV the alleles coding for GBSS of amylose-synthesizing clones derived from the amf mutant were investigated. Given the nature of the mutation, second site mutations would be expected in the genome of at least part of these clones and the *amf* mutation would still be in the GBSS gene if these clones were revertants. Our analyses revealed that in all investigated "revertant" clones the wild-type sequence was present. This led to the alternative conclusion that chimerism of wild-type and amylose-free tissues must have endured in the original mutant. Wild-type cells could have remained in the subepidermal layer of the original monoploid mutant, and alterations in tissue organization could have been induced by X-irradiation and aging, giving rise to apparent revertant sectors. At the sequence level chimerism was shown by applying direct sequencing of PCR fragments amplified from leave-DNA of a chimeric plant.

In the chapters II and III the applicability of the complementation system has already been shown. The advantages of transformation of amf stem segments with GBSS gene constructs via *A. rhizogenes* combined with iodine staining of root tips were further exploited to test the functionallity of semi-artificial TP sequences. The majority of the constructs described in chapter V were designed as controls to test the hypothesis of reinitiation of translation in the amf mutant. However, these control constructs did not result in functional GBSS preproteins and the question whether reinitiation indeed occurs remained unanswered. Still, the results of these experiments are interesting enough to be discussed in the light of requirements of TP sequences. A short TP, based on the sequence of a maize CAB preprotein was not able to transport GBSS into the plastid. This TP contained at positions 4-5 the subsequence Gly-Ser, which is probably responsable for the inactivity of the gene product. When the wild-type GBSS sequence is altered at positions 4-5 by substituting Gly-Ser for Ile-Thr, no amylose synthesis was found either. When the short CAB TP-like sequence replaces a part of the GBSS TP resulting in a TP of 99 residues, the gene product is active. The latter activity was abolished when small parts of the inserted CAB TP-like sequence were removed.

The last experimental chapter describes the sequence- and functional analysis of a gene located closely behind the GBSS gene. It is a unique member of the ß-1,3-glucanase and ß-1,3-1,4-glucanase gene family and is also present in tomato. Although initially some indications pointed towards a gene which could possibly be active, the majority of experimental information led to the conclusion that this gene presumably is a pseudogene. Neither in potato nor in tomato activity of this gene was found. In addition, the codon usage and the structure of the 3' part of the coding sequence suggest that this gene is not expressed.